Improving Acute Care Using Coagulation Mixing Studies

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The Fritsma Factor, Your interactive Hemostasis Resource℠
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Surgeons and physicians order PT and PTT assays to predict bleeding risk, often generating isolated, unexplained prolonged PTs or PTTs. In follow-up, the laboratory scientist mixes patient plasma with normal plasma and repeats the assay on the mixture. Mixing studies distinguish between coagulation factor deficiencies and plasma inhibitors, and may be provided by laboratory practitioners at community hospitals and acute care facilities. The information gained from mixing studies is an essential first step in the diagnosis of many hemostatic abnormalities. During this presentation, we discuss the importance of mixing studies, describe how they are performed, and show how their results contribute to diagnosis.
The participant...

• Lists the clinical applications for a PTT mixing study.
• Lists the steps to perform a PTT mixing study.
• Explains why the mixing study is an acute care assay.
• Correlates mixing study results with lupus anticoagulant and specific inhibitor testing.
Mixing Study: An Acute Care Assay
Differentiates a coagulopathy from a specific inhibitor or lupus anticoagulant

Case: 32-yo Female
Pre-op Screen

• Six weeks post-partum
• Easy bruising, frequent nosebleeds, vaginal bleeding
Pre-op Screen
32-yo Female, 6 Weeks Post-partum

<table>
<thead>
<tr>
<th>Assay</th>
<th>Patient</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGB</td>
<td>11.8 g/dL</td>
<td>12–15 g/dL</td>
</tr>
<tr>
<td>PT</td>
<td>12.4 s</td>
<td>9.8–12.6 s</td>
</tr>
<tr>
<td>PTT (APTT)</td>
<td>42.5 s</td>
<td>25–35 s</td>
</tr>
<tr>
<td>PLT count</td>
<td>310,000/µL</td>
<td>250–450,000/µL</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>320 mg/dL</td>
<td>220–498 mg/dL</td>
</tr>
</tbody>
</table>

Isolated, prolonged PTT response? Go to 1:1 PTT mix
Rule Out Heparin, DOACs

<table>
<thead>
<tr>
<th>Assay</th>
<th>Patient</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin time</td>
<td>14 s</td>
<td>&lt;21 s</td>
</tr>
<tr>
<td>Anti-Xa</td>
<td>0.0</td>
<td>0.4–0.7 U/mL</td>
</tr>
</tbody>
</table>

- Inpatient—unrecorded UFH flush of vascular access device
  - neutralize w/ Hepsorb (polybrene) or Hepzyme, proceed
- Outpatient: direct oral anticoagulant (DOAC): discontinue
  - Dabigatran, direct thrombin inhibitor, thrombin time markedly prolonged
  - Direct anti-Xa like rivaroxaban elevates anti-Xa, may prolong PTT
Direct Oral Anticoagulants (DOACs)

• Anti-Xas
  – Rivaroxaban; Xarelto
  – Apixaban: Eliquis
  – Edoxaban; Savaysa
  – Betrixaban; now at the FDA

• Direct thrombin inhibitor (DTI)
  – Dabigatran; Pradaxa

• If no A/C, perform 1:1 PTT mix to differentiate factor deficiency from factor-specific inhibitor or the “non-specific inhibitor,” lupus anticoagulant (LA, LAC)
PTT Mixing Study: Cheap and Simple

• Start within 2 hours of collection to avoid specimen degradation
  – Factors V (FV) and VIII (FVIII) deteriorate
  – Ensure patient plasma is platelet-poor, < 10,000/uL
  – If not, platelet factors released: PF4, FV

• Mix patient plasma 1:1 with pooled normal plasma (NP) and perform immediate PTT on mixture

• If PTT of 1:1 mix “corrects” to ≤10% longer than NP PTT
  – Factor deficiency? (But first you must incubate)

• No correction: 1:1 mix is >10% above NP PTT
  – Non-specific inhibitor, usually LA
  – Specific inhibitor (anti-FVIII), usually requires 37°C incubation
PTT Mixing Study

- Patient plasma (PTT 42.5 s) + Normal plasma (PTT 30 s) = 1:1 mix

Manufacturer’s value confirmed by laboratory QA supervisor
PTT Mixing Study
Using 10% Rule

100 uL PTT reagent

1:1 mix

1:1 mix + PTT rgt

100 uL CaCl₂

1:1 mix + PTT rgt + CaCl₂

PTT

≤33 s: Correction

>33 s: No correction
1:1 PTT Mix with Incubation

- PTT of immediate mix $\leq$10% longer than NP
  - Correction: factor deficiency? But first…
  - Incubate 1:1 mix 1–2 hours and repeat
- Correction after incubated mix = factor deficiency
- No correction: PTT remains >10% above NP
  - Specific inhibitor such as anti-FVIII
    - IgG$_4$: Temp dependent, usually requires incubation
    - However, some inhibitors neutralize FVIII within 10 min
    - May detect in immediate mix
1:1 PTT Mix with Incubation

- Reflex to incubation if unincubated mix corrects
- Must also incubate NP
- Compare mix PTT to incubated NP PTT
- May also detect temperature-dependent LA
  —~15% of LAs are temperature-dependent

**37°C Incubated 1:1 PTT Mix**

- Patient plasma: PTT 42.5 s
- Incubated PTT 35 s
- 1:1 mix: incubate 1–2 h, repeat PTT

PTT of Mix:
- ≤38.5 s: Correction
- >38.5 s: No correction
## Mixing Study Result

### 32-yr Female, 6 Weeks Post-partum

<table>
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<tr>
<th>Assay</th>
<th>Result (s)</th>
<th>RI</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTT</td>
<td>42.5</td>
<td>25–35 s</td>
<td>Confirms previous PTT</td>
</tr>
<tr>
<td>PTT/control 1:1 mix</td>
<td>32.1</td>
<td>Control 30 s</td>
<td>Commercial platelet-free control plasma (NP)</td>
</tr>
<tr>
<td>immediate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTT/control 1:1 mix</td>
<td>37.3</td>
<td>Control 35 s</td>
<td>Incubate both 1:1 mix and NP</td>
</tr>
<tr>
<td>1–2 h at 37°C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Conclusion:

Both immediate *and* incubated mix PTTs correct, suspect factor deficiency, arrange for factor assays and von Willebrand disease profile.
### Factor Assay Results

**32-yo Female, 6 Weeks Post-partum**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Result</th>
<th>RI</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII</td>
<td>32%</td>
<td>50–150%</td>
<td>?</td>
</tr>
<tr>
<td>IX</td>
<td>92%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XI</td>
<td>131%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XII</td>
<td>113%</td>
<td></td>
<td>XII, HMWK &amp; PK deficiency not associated with bleeding</td>
</tr>
<tr>
<td>HMWK</td>
<td>ND</td>
<td>65–135%</td>
<td></td>
</tr>
<tr>
<td>PK</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
PTT reagent: Ca^{++}, particulate activator, phosphatidyl serine; test prolonged by XII, PK, HMWK, XI, IX, VIII, X, V, prothrombin, Fg deficiency; heparin Rx, LAC

PT reagent: tissue factor, Ca^{++}, phosphatidyl serine; prolonged by VII, X, V, prothrombin, Fg deficiency; coumadin Rx

Figure courtesy of Margaret G. Fritsma, Rodak’s Hematology, 5th Edition, 2015
# PT and PTT Results in Inherited Single-factor Coagulopathies

<table>
<thead>
<tr>
<th>PT</th>
<th>PTT</th>
<th>Single Factor Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long</td>
<td>Normal</td>
<td>VII</td>
</tr>
<tr>
<td>Long</td>
<td>Long</td>
<td>X, V, II, and fibrinogen(^1)</td>
</tr>
<tr>
<td>Normal</td>
<td>Long</td>
<td>VIII, IX, XI(^2)</td>
</tr>
</tbody>
</table>

\(^1\)PT & PTT prolonged when fibrinogen is <100 mg/dL, perform fibrinogen assay

\(^2\)Contact factor deficiencies XII (1–3% prevalence), prekallikrein (PK, Fletcher), or high molecular weight kininogen (HMWK, Fitzgerald) also prolong PTT results, but no bleeding
PTT Mix: Why Does This Work?

- Hypothetical 20% F VIII level prolongs PTT
  - PTT rgts calibrated to prolong at 30–40% FVIII, IX, XI
- Add NP with established 100% factor level
  - 1:1 mix, average of 100% and 20% = 60%, PTT corrects
- Hypothetical anti-FVIII or lupus anticoagulant
  - With typical avidity, retains its ability to prolong the mix

\[
\text{Patient} \quad \begin{array}{c}
20\% \\
\text{FVIII}
\end{array} \quad + \quad \begin{array}{c}
100\% \\
\text{FVIII}
\end{array} = \begin{array}{c}
1:1 \text{ Mix:} \\
60\% \\
\text{FVIII}
\end{array}
\]
52-yo Athletic Female

Pre-op screen for total hip replacement
## 52-yeo Athletic Female
Screen Prior to Hip Replacement Surgery

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGB</td>
<td>14.1 g/dL</td>
<td>12–15 g/dL</td>
</tr>
<tr>
<td>PT</td>
<td>11.2 s</td>
<td>9.8–12.6 s</td>
</tr>
<tr>
<td>PTT</td>
<td>58 s</td>
<td>25–35 s</td>
</tr>
<tr>
<td>PLT</td>
<td>170,000/μL</td>
<td>150–400,000/μL</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>410 mg/dL</td>
<td>220–498 mg/dL</td>
</tr>
</tbody>
</table>

Patient reports no bleeding or bruising, no thrombosis
Isolated Prolonged PTT: Differential

- Could be nothing: 5% of normals exceed limit
- Preanalytical variable: green or lavender-closure tube, hemolysis, lipemia, clotted specimen
- Outpatient: dabigatran
- Inpatient: unreported UFH
- Congenital single factor deficiency: VIII, IX, or XI, hemophilia A, B, or C with bleeding, VWD
- Congenital FXII, PK, or HMWK without bleeding
- Acquired FVIII inhibitor with severe bleeding
  - “Acquired hemophilia”
- Lupus anticoagulant (LA)
### 52-yo Female PTT Mixing Study

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>17 s</td>
<td>RI: &lt; 21 s, rules out dabigatran</td>
</tr>
<tr>
<td>PTT</td>
<td>58 s</td>
<td>RI: 25–35 s</td>
</tr>
<tr>
<td>PTT NP</td>
<td>28 s</td>
<td>Correction if &lt; 30.8 s (10%)</td>
</tr>
<tr>
<td>1:1 mix</td>
<td>35 s</td>
<td>25% over NP = no correction</td>
</tr>
</tbody>
</table>

What is the next step?
Acute Care Mixing Study Algorithm

Isolated prolonged PTT

TT long

Heparinase or polybrene, recheck TT

If TT long, dabigatran, stop here

TT

TT normal

Patient & NP 1:1 mix

Correction

LA profile

Correction

Factor assay

Incubated patient & NP 1:1 mix

No correction

FVIII inhibitor
Mixing Study Considerations

- Preanalytical variables
  - Anti-Xa rivaroxaban, apixaban, edoxaban prolong PT, PTT
  - Dabigatran and UFH prolong PTT
  - Clotted, hemolyzed, lipemic specimen
  - Underfilled tube, wrong anticoagulant
  - Must be platelet-poor, <10,000/uL patient and NP

- Heparinase neutralizes ≤1 unit/mL UFH

- 15% of anti-FVIII inhibitors are detected in immediate mix

- 15% of LAs require incubation

- Weak LAs may be missed in 1:1 mix
  - Select a more LA-sensitive PTT reagent or prepare a 4:1 mix
The “LA Cofactor’ Effect

- Initial PTT = 48 s, RI 25–35; 1:1 mix prolongs to 54 s
- Prothrombin binds LA, slows coagulation?
- LA potentiates clotting via annexin V, the mix reverses potentiation?

Normal Plasma Source?

• Home brew: ~pool 20 normal plasmas, male ≈ female
  – Ensure plasma is platelet-poor; < 10,000/uL; PTT ≅ mean of RI
  – Ensure NP has ~100% of all factors, especially VIII, IX, and XI
  – Elevated FVIII causes false negative results
  – Screen each for LA, specific factor inhibitors. HBV, HCV, HIV
  – Aliquot and freeze

• Or purchase commercial plasma
  – GMP & frozen meets all criteria
  – Lyophilized plasma acceptable when validated
    • Processed with stabilizers

What Limit Defines Correction?

No Consensus; Fritsma Factor Quick Question Answers

• Limits based on fixed PTT value such as reference interval
  – 1:1 mix within RI upper limit (95% or 99% confidence interval, 39%)
  – 1:1 mix within RI upper limit + 5 seconds (8%)

• Limits based on the pooled normal plasma PTT value
  – 1:1 mix within NP PTT value + 5 seconds (14%)
  – 1:1 mix within NP PTT + 10% (32%)

• Rosner or Chang limit formula using patient, NP, and 1:1 mix
  – Rosner formula produces a ratio
  – Chang’s formula produces % deviation, requires incubation of patient plasma

• Other (7%): combination of RI and Rosner
  – Dedicated RI for mix
Chang Index: Limit Based on % Correction

\[
\text{% Correction} = \frac{\text{Patient PTT} - \text{1:1 mix PTT}}{\text{Patient PTT} - \text{NP PTT}} \times 100
\]

\[
\text{% Correction} = \frac{42.5 - 32.1}{42.5 - 30} = \frac{10.4}{12.5} = 0.83 = 83\%
\]

Factor Deficiency = \geq 75%
Inhibitor = < 75%

% Correction verified by local laboratory

Rosner Index

\[
\text{Rosner Index} = \frac{1:1 \text{ mix PTT} - \text{NP PTT}}{\text{Patient PTT}} \times 100
\]

\[
\text{Rosner Index} = \frac{32.1 - 30}{42.5} \times 100 = 4.9
\]

Inhibitor Correction

- \( \geq 11 \)
- \(< 11 \)

Rosner index validated by local laboratory

Slightly more conservative than 10%

59-yo Male
Former Hockey Player
Total knee replacement preop labs
59-yo Male Former Hockey Player
Screen Prior to Knee Replacement Surgery

<table>
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<tbody>
<tr>
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</tr>
<tr>
<td>PT</td>
<td>11.2 s</td>
<td>9.8–12.6 s</td>
</tr>
<tr>
<td>PTT</td>
<td>38 s</td>
<td>25–35 s</td>
</tr>
<tr>
<td>PLT</td>
<td>310,000/µL</td>
<td>150–400,000/µL</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>390 mg/dL</td>
<td>220–498 mg/dL</td>
</tr>
</tbody>
</table>

Patient reports no bleeding or bruising, no thrombosis
When to Perform Mixing Study

- Any PTT > RI upper limit
- Any PTT > RI upper limit + 5 seconds
- Any PTT > RI upper limit with consult
  - Is patient bleeding or clotting?
  - Possible “weak” LA: use 4:1 mix
  - Lupus sensitive PTT reagent
  - Factor sensitive PTT reagent

## 59-yo Male Former Hockey Player

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<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>17 s</td>
<td>RI: &lt; 21 s, rules out dabigatran</td>
</tr>
<tr>
<td>Anti-Xa</td>
<td>0.0</td>
<td>0.4–0.7 U/mL, r/o direct anti-Xa</td>
</tr>
<tr>
<td>PTT</td>
<td>38 s</td>
<td>RI: 25–35 s</td>
</tr>
<tr>
<td>PTT NP</td>
<td>31 s</td>
<td>Correction if &lt; 34.1 s (10%)</td>
</tr>
<tr>
<td>1:1 mix</td>
<td>35 s</td>
<td>Correction? No correction?</td>
</tr>
</tbody>
</table>

**What is the next step?**
59-yo Male Former Hockey Player
Clinical Consult

• Consult: if no medical conditions go on to TKR
• Prior thrombotic events (VTE)
  – Perform mix using 4:1 patient plasma to NP
  – Or choose PTT reagent that is LA-sensitive
• If anatomic bleeding, test for FVIII, FIX, FXI
  – Vitamin K deficiency: factor VII
  – Renal insufficiency
  – Liver disease (factor V), malignancy, VWD
2 YO Hemophilic Boy

Bleed into knee and ankle
# 2-yo Hemophilic Boy

<table>
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</thead>
<tbody>
<tr>
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</table>

Inflamed, swollen knee and ankle
Mixing Study Result
2-yo Hemophilic Boy

<table>
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<th>Result</th>
<th>RI</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTT</td>
<td>65 s</td>
<td>25–35 s</td>
<td>Confirms previous PTT</td>
</tr>
<tr>
<td>PTT/control 1:1 mix immediate</td>
<td>33.5 s</td>
<td>NP 30 s</td>
<td>Correction (ambiguous)</td>
</tr>
<tr>
<td>PTT/control 1:1 mix 1 h at 37°C</td>
<td>47.9 s</td>
<td>NP 35 s</td>
<td>Control is incubated alone and with mix</td>
</tr>
</tbody>
</table>

Conclusion: Anti-FVIII inhibitor
Factor VIII Assay

- Dilute plasma 1:10
- Add factor VIII-depleted reagent plasma 1:1
- Add PTT reagent, incubate 3 minutes
- Add CaCl₂, record interval to clot formation
- Compare result in seconds to calibration curve
Factor VIII Assay Dilutions
Parallelism Indicates No Inhibitor

<table>
<thead>
<tr>
<th>Plasma Dilution</th>
<th>Seconds</th>
<th>Raw Factor VIII Activity</th>
<th>Computed Factor VIII Activity (× dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10 “undiluted”</td>
<td>90 s</td>
<td>20%</td>
<td>20%</td>
</tr>
<tr>
<td>1:20</td>
<td>104 s</td>
<td>10%</td>
<td>20% (parallel)*</td>
</tr>
<tr>
<td>1:40</td>
<td>107 s</td>
<td>5%</td>
<td>20% (parallel)</td>
</tr>
<tr>
<td>1:80</td>
<td>110 s</td>
<td>2.5%</td>
<td>20% (parallel)</td>
</tr>
</tbody>
</table>

* <10% difference from undiluted indicates parallelism, no inhibitor
# FVIII Assay Dilutions

non-Parallelism Indicates Inhibitor

<table>
<thead>
<tr>
<th>Plasma Dilution</th>
<th>Seconds</th>
<th>Raw Factor VIII Activity</th>
<th>Computed Factor VIII Activity (× dilution)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10 “undiluted”</td>
<td>80 s</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>1:20</td>
<td>93 s</td>
<td>8%</td>
<td>16%</td>
</tr>
<tr>
<td>1:40</td>
<td>107 s</td>
<td>5%</td>
<td>20%</td>
</tr>
<tr>
<td>1:80</td>
<td>108 s</td>
<td>4%</td>
<td>32%</td>
</tr>
</tbody>
</table>

* >10% difference from undiluted, rising = non-parallel, implies inhibitor

55-yo Male with Atrial Fibrillation

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<tr>
<td>PTT</td>
<td>159 s</td>
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</tr>
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55-yo Male with Atrial Fibrillation

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<td>159 s</td>
<td>25–35 s</td>
</tr>
<tr>
<td>TT</td>
<td>&gt; 150 s</td>
<td>&lt; 21 s</td>
</tr>
<tr>
<td>PTT/control 1:1 mix immediate</td>
<td>78 s</td>
<td>Control 30 s</td>
</tr>
<tr>
<td>PT/control 1:1 mix immediate</td>
<td>15.2 s</td>
<td>Control 12 s</td>
</tr>
</tbody>
</table>

What do you recommend?
If the PT is Prolonged

• Congenital deficiencies of II, V, VII, or X
  – PT and PTT long: II, V, X
  – PT only: VII, skip mixing and go to factor assay
  – Prevalence: 500,000–1:2,000,000
• Vitamin K deficiency: des-carboxy II, VII, and X
• Liver disease: PT prolongs before PTT due to des-carboxy II, VII, and X, reduced factor V
• Anti-Xa direct oral anticoagulants
  – Rivaroxaban, apixaban, edoxaban, betrixaban (phase 3)
Isolated Prolonged PTT: Summary

- Random benign prolongation, 95% CI
- Lupus anticoagulant: Prevalence of 1–3%
- Drug reaction producing transient LA
- Unrecorded heparin, dabigatran, oral anti-Xa
- Known hemophilic who fails FVIII concentrate Rx
- Hemorrhage or ecchymoses signal acquired coagulopathy; vitamin K deficiency, liver disease
- Specific inhibitor, anti-FVIII
  - Postpartum, malignancy
  - Autoimmune disorders, > 60-yo

Develop Mixing Study Reliability

- PTT reagent sensitivities to factors and to LA
  - 30–40% FVIII, FIX, FXI
  - Intermediate sensitivity to LA
- NP consistency: ~100% activity for all factors
- Consultation for equivocal patient results
- Employ consistent correction limit
DIY Local Mixing Studies—Why?

- Unexpected isolated prolonged PTT or PT may require immediate action
- Local results may immediately direct therapy
- Delayed specimen may deteriorate
- Forward mixing study results to ref lab to direct follow-up, for instance, LA workup or Bethesda titer
Bottom Line at the End (BLEAT)

The participant...

- Lists the clinical applications for a PTT mixing study.
- Lists the steps to perform a PTT mixing study.
- Explains why the mixing study is an acute care assay.
- Correlates mixing study results with lupus anticoagulant and specific inhibitor testing.
Thanks for listening!
Ya got any questions?

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